***Strongylocentrotus purpuratus* Fertilization Test**

1.0 OBJECTIVE

The purpose of the fertilization test with the sea urchin, *Strongylocentrotus purpuratus*, is to determine if seawater, porewater, sea surface microlayer, or other samples inhibit fertilization in eggs and sperm relative to gametes exposed to control or reference samples (US EPA 1995). The test may also be used to determine the concentration of a test substance that impairs fertilization.

2.0 EQUIPMENT

The following equipment is necessary to conduct the toxicity test at the Marine Pollution Studies Laboratory at Granite Canyon (MPSL). The word "clean" here and throughout this procedure means that the item has been cleaned according to the MPSL glassware cleaning procedures outlined in a separate SOP (MPSL SOP 1.3).

2.1 Urchin Collection and Culture

- Tanks, trays, or aquaria for holding adult sea urchins
- Air pump, airlines, and air stones

2.2 Test Initiation

- Squirt bottle filled with seawater
- 20-mL glass scintillation vials (leached in dilution water for 24 hours)
- 10-mL glass pipettes and pipettor
- Environmental chamber (15 ± 1°C, ambient laboratory illumination for 16 hours/day)
- Culture micropipettors and tips (10-1000 mL)
- Glass Pasteur pipettes and bulb
- Glacial acetic acid
- Hemacytometer
- Sedgwicke-Rafter counting cell
- Culture graduated cylinders
- 0.5 M KCl
- Syringe and 24-gauge needle for injecting urchins
- Control water (Granite Canyon seawater) and hypersaline brine

2.3 Test Termination

- Inverted compound microscope
- Data sheets
- Formaldehyde, 37% (concentrated buffered formalin) and volumetric pump
- Fume hood
- Counter

2.4 Water Quality

• Meters, probes and spectrophotometer for measuring pH, dissolved oxygen, salinity, and ammonia
• Thermometers (glass spirit thermometer)
• Graduated pipettes and 30-ml glass vials
• Gloves and appropriate safety gear (see MPSL lab safety manual)

2.5 Dilution Water

Dilution water consists of ambient Granite Canyon seawater, filtered to 1 µm, at ambient salinity (33-34‰). This water is used to prepare eggs and sperm for toxicity tests, and for diluting test solutions.

3.0 EXPERIMENTAL DESIGN

Aquatic toxicity tests can be used as screening tools or as part of more comprehensive studies to assess water quality. Careful consideration must be given to site characteristics, reference site selection, field replication, choice of synoptic measures, seasonal factors, and comprehensive planning and peer review to determine that study designs are adequate to meet program objectives.

This laboratory toxicity test consists of five replicate scintillation vials for each sample. Samples are occasionally diluted into several concentrations. Vials are arranged randomly, and each receives about 1000 eggs and concentration of sperm determined by a fertilization pre-test. The quality of test gametes and testing conditions is determined through concurrent testing of reference toxicants (positive controls), and seawater and hypersaline brine controls (negative controls). Testing of reference sites is recommended to demonstrate the suitability of test samples in the absence of toxic contaminant concentrations. Dissolved oxygen, pH, salinity, ammonia, and temperature are measured at the beginning of the exposure.

4.0 SAMPLE PREPARATION

Because of the 48-hour holding time, tests will generally be initiated on the same day as sample receipt. Label scintillation vials as indicated on the randomization sheet generated for the test. Determine the salinity of the test solutions. Be sure to homogenize all samples prior to measuring salinity. Samples with salinities below 32‰ are adjusted to 34‰ with hypersaline brine (see salinity adjustment data sheet).

Using the random number sheet, aliquot 5 mL of sample to scintillation vials. Minimize sample exposure to sunlight (never leave samples in direct sunlight), and schedule loading times to avoid prolonged sample exposure to temperatures above 15°C.

5.0 CONTROLS

5.1 Seawater and Brine Controls (Negative Controls)

A seawater control consisting of 1-µm filtered Granite Canyon water should accompany each batch of samples. If any salinity adjustments were made, brine controls must also be prepared. The brine control must contain the same amount of brine as the lowest salinity sample (see salinity adjustment data sheet).

5.2 Reference Toxicant Tests (Positive Controls)

Conduct a concurrent reference toxicant test on a monthly basis. The reference toxicant test is for a similar exposure time, and provides data on the relative sensitivity of each batch of urchin gametes. Prepare a stock solution of 10,000 µg Cu/L by weighing 0.0268 grams of copper chloride ($CuCl_2$), and pouring the weighed solid into one liter of distilled water in a plastic volumetric flask. Reference toxicant solutions should be four replicates of 0, 5.6, 10, 18, 32, and 56 µg/L. Other concentrations may be added between these if greater precision is desired for quality control chart purposes. Prepare concentrations according to dilution schedule. Start with the control solutions and progress to the highest concentration to minimize contamination. Use plastic volumetric flasks and beakers to reduce loss of copper to container walls.

6.0 TEST INITIATION

6.1 Test Organisms

Newly released eggs and sperm of the sea urchin, *S. purpuratus* are used in the fertilization toxicity test. Animals may be collected in the field or obtained from a commercial supplier. *S. purpuratus* is distinguished from *S. franciscanus*, a less common cogener, by its purple to occasionally green color. Urchins can be maintained easily in aquaria or other tanks provided with running seawater and aeration. Urchins will eat a wide variety of marine algae, but prefer giant kelp, *Macrocystis pyrifera*. To ensure year-round spawning, broodstock are held at ambient seawater temperature, in complete darkness. Tanks should be cleaned weekly during feeding, and ambient salinity should be maintained. The sex of the urchins may be determined at the time of spawning. Once these animals are re-conditioned they may be spawned for use in later toxicity tests.

6.2 Test System

Five replicates are used for all treatments. Each replicate contains 5 mL of test solution. A hypersaline brine control is necessary if any salinity adjustments are required. The brine control should contain the amount of brine equal to that used to adjust the sample with the lowest salinity. Make the brine control by first adding enough Nanopure® or distilled water to adjust Granite Canyon dilution water to a salinity equal to that of the lowest salinity sample. Then adjust this sample back up to 34 ± 2‰ using an appropriate amount of brine (see salinity adjustment data sheet).

6.3 Collection and Preparation of Gametes

To perform the test repeatedly, quality gametes must first be collected, and then diluted to the appropriate density for addition to the test vials.

6.3.1 Spawning of Urchins

• Place ten urchins on several layers of paper towels on a clean surface.

• Inject each urchin with 0.5 mL of 0.5 M KCl in the anal pore. Alternately, KCl can be injected into the soft tissue surrounding the Aristotle's lantern. Gently shake the animals once or twice to stimulate gamete release. Wipe the needle before injecting the next urchin, to avoid uncontrolled fertilization. Keep urchins wet using a seawater-filled wash bottle.

• Re-inject with 0.5 mL of 0.5 M KCl, any urchins that have not spawned after 10 minutes.

• Females release orange-colored eggs, and males release cream-colored sperm.

• Collect gametes before 30 minutes have elapsed. Sperm must be used within 4 hours.

6.3.2 Gamete Quality

• Eggs must be inspected for uniformity and roundness. Select only females with uniformly round eggs that lack follicles. Do not use a batch of eggs if a high proportion of germinal vesicles are present or if eggs are released from females in large clumps.

• Sperm should be extracted from the urchins with a 100 µL pipette. Suck sperm from the dorsal side of the urchin in 25 µL aliquots and place it in a small beaker. Keep the beaker on ice to minimize sperm motility.

• Eggs should be washed off the urchins with a squirt bottle filled with 1-µm filtered seawater.

• Place one drop of eggs onto a well slide and add a small amount of sperm to test fertilization. Check for a fertilization membrane. If no fertilization membrane is present, isolate new eggs.

• Place the eggs and sperm in the constant temperature room (12°C) until ready for counting.

6.3.3 Fertilization of Gametes

Use the urchin fertilization worksheet to determine the appropriate sperm and egg dilution factors. Sperm and eggs are combined based on worksheet equations. A sperm to egg ratio pretest is conducted prior to definitive toxicity test. This pretest determines the appropriate sperm to egg ratio.

7.0 TEST TERMINATION

To terminate the test, use a toxic dispenser to add 1.0 mL 37% buffered formaldehyde to each vial to give a final formaldehyde concentration of 4% formalin. Gently shake containers to mix. As an alternate fixative, 0.5 mL of 1.0% gluteraldehyde may be added to each test container. Test containers may now be capped and stored for later evaluation.

8.0 DATA COLLECTION AND TABULATION

Count 100 eggs per sample using the hand counter. Use one key to indicate fertilized eggs (embryos) and another to indicate unfertilized eggs. Eggs are considered fertilized if an elevated membrane is present (see protocol for figures and guidance). Calculate percent fertilization for each control replicate test. Test acceptability is 70% fertilization in the seawater control.

9.0 DATA HANDLING AND TEST ACCEPTABILITY

Immediately after test termination, check the data sheet to determine whether dilution water and salinity controls have acceptable fertilization (mean of 70% or greater). Tests with temperature, salinity, or dissolved oxygen measurements outside the specified ranges, may be considered conditionally acceptable based on the project officer's best professional judgment. Acceptable temperatures are 12 ± 1°C; acceptable dissolved oxygen concentration is 60-100% saturation.

10.0 REFERENCES

U.S. Environmental Protection Agency. 1995. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. Office of Research and Development. EPA/600/R-95/136. August 1995

11.0 TEST SUMMARY

Species:	*Strongylocentrotus purpuratus*
Test Duration:	20 minutes
Endpoint:	fertilization
Organism Source:	Wild-caught adult broodstock that has been maintained in the laboratory in constant darkness and flow through seawater
Test Salinity:	Ambient ± 2‰
Test Temperature:	12± 1°C
Dilution water:	1 µm filtered seawater at 12°C.
Replication:	5 replicates per sample
Test Containers:	20 mL glass scintillation vials
Loading:	Approximately 1000 eggs and no more than 3.36×10^6 sperm
Water Quality:	dissolved oxygen, pH, salinity, ammonia, temperature
Reference Toxicant:	copper chloride ($CuCl_2$)
Acceptability Criteria:	Controls: ≥70%